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DNA barcoding is an effective tool for differentiating *Pisolithus* species from Macedonia

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Abstract — With the aim of clarifying the number of *Pisolithus* species in Macedonia, molecular analysis was carried out on seven specimens collected in oak forests in Macedonia during 1987 and 2005. New sequences were compared with previously published sequences included in public databases. Three *Pisolithus* species are identified in Macedonia: *P. arhizus*, *P. capsulifer*, and *P. tinctorius*.

Key words — gasteromycetes, *Boletales*, ITS nrDNA, phylogeny

Introduction

Pisolithus Alb. & Schwein. is a genus widely distributed across temperate to tropical regions and forming ectomycorrhiza with many different woody plants (Marx 1977, Chambers & Cairney 1999). Phylogenetic studies conducted particularly in the last decade have pointed to the presence of several species in this genus (Anderson et al. 1998, 2001; Martin et al. 1998, 2002; Gomes et al. 2000; Díez et al. 2001) that was for a long time considered monotypic (Coker & Couch 1928, Cunningham 1942, Pilát 1958). Recently several new species have been recognized, such as *P. orientalis* Watling et al., associated with *Pinus kesiya* (Phosri et al. 2012); the new combination *P. capsulifer* (Phosri et al. 2012); *P. calongei* M.P. Martín et al., mycorrhizal with *Cistus* (Martín et al. 2013);

P. croceorrhizus P. Leonard & McMullan-Fisher associated with *Myrtaceae* (Leonard et al. 2013); a close relative of *Pisolithus* sp. 10 Martin et al. (2002); and *Pisolithus* sp. 12/13 (Leonard et al. 2013). These new analytical methods have confirmed previous hypotheses based on macroscopic and microscopic variations proposed by Bronchart et al. (1975), Calonge & Demoulin (1975), and Demoulin & Dring (1975). The only species recognized in Macedonia based on morphological features is *Pisolithus arhizus* (Karadelev et al. 2008), which is included in the Macedonian Red List of Fungi (Karadelev & Rusevska 2013). In order to determine the precise number of *Pisolithus* species in Macedonia, we decided to analyze the internal transcribed spacer region of nuclear ribosomal DNA (ITS nrDNA) obtained from several different specimens collected in Macedonia.

Materials & methods

Morphological studies

Dried samples used in this study are deposited in the Macedonian Collection of Fungi, Mycological Laboratory, Faculty of Natural Sciences and Mathematics, Sts. Cyril and Methodius University, Skopje, Macedonia (MCF). Morphological identification of the specimens has been made according to Jülich (1984), Pegler et al. (1995), Calonge (1998), Krieglsteiner (2000), Sarasini (2005), Knudsen & Vesterholt (2012). Microscopic measurements and photographs were made from tissue sections mounted in 5% KOH and examined at magnifications up to 1000× with an LW Scientific microscope. SEM was carried out after coating dried glebal samples in gold with Balzers SCD 004 sputter coater with a Hitachi S-3000N SEM. 30 spores were measured from each collection. The species distribution map was generated in ArcGIS 10.1 based on Digital Elevation Model (ASTERGDEM) (http://asterweb.jpl.nasa.gov/gdem.asp, 09. 2012).

Molecular analyses

DNA extraction, amplification, and sequencing of the ITS regions were made from glebal tissue of dry field collected basidiomes using the protocols described in Phosri et al. (2009). The ITS region, including the 5.8S rDNA the ribosomal RNA gene cluster and small flanking parts of SSU and LSU genes, was amplified by PCR with ITS1F and ITS4 primer pair (White et al. 1990, Gardes & Bruns 1993) according to the cycling protocol in Martín & Winka (2000). Five µl aliquots of PCR products were verified by gel electrophoresis in 2% Pronadisa D-1 Agarose (Lab. Conda, Spain). The amplified products were purified with QIAquick gel PCR purification kit (QAGen, Valencia, California) and sequenced by Macrogen, Inc. (South Korea). The new *Pisolithus* sequences were aligned with 99 sequences obtained from Phosri et al. (2012) and analyzed using a heuristic search in PAUP 4.0b10 (Swofford, 2003) and under a Bayesian approach (Huelsenbeck et al. 2000, Larget & Simon 1999) assuming a HKY+G

Fig. 1. Phylogenetic tree (one of 100 most parsimonious trees) obtained with a parsimony analysis under heuristic search of ITS nrDNA sequences of *Pisolithus* specimens (indicated with the MCF herbarium number) and sequences from Phosri et al. (2012), with sequences of *Suillus luteus* and *Scleroderma citrinum* as outgroup.

GU373495 Suillus luteus
FM213344 Scleroderma citrinum
C
100
AF415227 Shorea macropera, Malaysia
AF415226 Shorea macropera, Malaysia
P. aurantioscabrosus (P. species11)
AY756113 Vateria indica, India
P. indicus (P. species 13)
100
AF003915 Afzelia sp., Kenya
AF228653 Afzelia sp., Kenya
P. species 1
A I
FR748119 Shorea sp., Thailand
AB099921 Shorea roxburgii, Thailand
100
AB099920 Shorea roxburgii, Thailand
FR748120 Thailand
AB099922 Dipterocarpus alatus, Thailand
P. abditus (P. species 12)
AF003914 Eucalyptus camaldunensis, Kenya
AF004733 Eucalyptus sp., New South Wales
AF374719 Eucalyptus calophylla, Australia
99
AF004734 Eucalyptus sp., New South Wales
AF440866 Eucalyptus plantation, China
AF374665 Eucalyptus patens, Australia
P. marmoratus (P. species 2)
83
AF228641 Cistus ladanifer, Spain
FR748140-FR748142 (3 clones) Cistus sp., Spain
100
AF228642 Cistus ladanifer, Spain
AF228643 Cistus ladanifer, Spain
AF228644 Cistus ladanifer, Spain
P. calongei (P. species 3)
AF374624 Acacia sp., Australia
P. species 10
AF347700 Eucalyptus sp., Brazil
AF004735 Eucalyptus sp., New South Wales
AF142991 Eucalyptus sp., Brazil
AF228657 Eucalyptus sp., Morocco
AF140547 Eucalyptus sp., Brazil
AF374704 Eucalyptus dunni, Brazil
56
66
U62666 Eucalyptus citriodora, Brazil
AF374661 Eucalyptus camaldunensis, Australia
AF374708 Eucalyptus sp./Acacia sp., Australia
AF004732 Eucalyptus sp., New South Wales
P. microcarpus (P. species 9)
AF270786 Eucalyptus sp., Australia
AF374622 Acacia holosericea, Senegal
FR748122 Eucalyptus camaldunensis,Thailand
FR748123 Eucalyptus camaldunensis,Thailand
87
AF228656 Eucalyptus sp., Spain
AF228655 Eucalyptus sp., Morocco
AF228654 Eucalyptus sp., Morocco
AB099918 Thailand
FR748121 Eucalyptus camaldunensis,Thailand
AB099909 Eucalyptus camaldunensis,Thailand
B 99
AF416589 Eucalyptus tereticornis, India
AF440868 Eucalyptus sp., China
AF374675 Eucalyptus sp., Queensland
AF004736 unknown potential host, New South Wales
AF004737unknown potential host, New South Wales
FR748124 Eucalyptus camaldunensis, Thailand
AJ629887 Thailand
FR748125 Eucalyptus sp., Hawaii, USA
FR748127 Melaleuca sp., Thailand
AB099911 Thailand
AF374638 Acacia sp./Mangium sp., Malaysia
FR748126 Eucalyptus sp., Australia
P. albus complex
(P. species 7)
AF374646 Eucalyptus globulus, Australia
P. species 10
AF440867 Eucalyptus plantation, China
P. microcarpus (P. species 9)
AF003916 Pinus caribea, Kenya
AF228645 Quercus ilex/Q. coccifera, Spain
AF228646 Quercus ilex/Cistus ladanifer, Spain
FR748138 Pinus sp., Spain
AF143234 Unknown potentital host, France
58
AF143233 Pinus sp., USA
05MCF5506
FR748139 Quercus sp., USA
AF228647 Pinus caribea, Kenya
P. tinctorius (P. species 6)
93
AF374625 Pinus kesiya, Thailand
AF374679 Eucalyptus sp., China
AF374711 Pinus sp./Eucalyptus sp., China
AB099919 Pisolithus sp., Thailand
FR748149 Pinus kesiya, Thailand
54
FR748147Pinus kesiya, Thailand
FR748146 Pinus kesiya, Thailand
AB099845 Pisolithus sp., Thailand
FR748145 Pinus kesiya, Thailand
FR748148 Pinus kesiya, Thailand
FR748143 Pinus kesiya, Thailand
FR748144 Pinus kesiya, Thailand
P. orientalis (P. species 5)
A II
FM213365 Pinus sp., Spain
AF228648 Quercus ilex/Q. coccifera, Spain
FR748128 (clone a),FR748131(clone d) Will Wood, Sardinia, Italy
02MCF9560
05MCF5324
87MCF7741
FR748130 (clone c) Will Wood, Sardinia, Italy
98MCF4331
FR748129 (clone b) Will Wood, Sardinia, Italy
AF228649 Quercus ilex, Spain
AF228651 Quercus ilex, Spain
54
AF228650 Pinus halepensis/Quercus coccifera, Spain
AF228652 Pinus halepensis, Spain
FR748132 (clone f) Will Wood, Sardinia, Italy
FR748133 Pinus sp., Spain
AF374627 Pinus sp. South Africa
P. arhizus (P. species 4)
AF374629 Pinus pumila/Betula ermanii, Japan
UDB001206 Pinus forest, Sweden
78
03MCF3428
FR748134 unknown potential host, England
02MCF3355
FR748135 unknown potential host, England
FR748138 mixed forest, France
FR748136 Pinus sylvestris/Pinus contorta, England
P. capsulifer (P. species 14)

– 5 changes

model with MRBAYES 3.0 (Huelsenbeck & Ronquist 2001), as described in Phosri et al. (2012). The phylogenetic tree was visualized in Treeview (Page 1996) and edited in Adobe Illustrator CS3. The names of clades and subclades follow Phosri et al. (2009).

Results

Genomic DNA concentration ranged from 1.5 to 15 ng/µl. The ITS region was successfully amplified and sequenced from 7 collections of dried *Pisolithus* basidiomes using ITS1F/ITS4 primer pair. After purification of the amplified product, the DNA concentration was greater than 20 ng/µl, and sequenced directly. The seven new sequences obtained in this study have been submitted to the international database (EMBL).

The phylogenetic tree (Fig. 1) obtained under heuristic search has similar topology with the 50% majority-rule tree of the Bayesian analysis (data not shown). New sequences are well grouped in linage AII (Phosri et al. 2012), but in three main clades, each corresponding to one *Pisolithus* species.

One sample grouped in the *P. tinctorius* clade. Four *Pisolithus* records that were placed in the *P. arhizus* clade were recorded from three different localities: Pogana (kermes oak shrubland), Galichica Mountain (Macedonian oak forest), and Skopska Crna Gora Mountain (oak–hornbeam forest). Two *Pisolithus* collections, both originating from Bistra Mountain, were placed in the *P. capsulifer* clade.

Pisolithus tinctorius (Pers.) Coker & Couch,
Gasteromycetes East. U.S.: 170. 1928. Fig. 2

Basidiomes pyriform with almost ellipsoid head, slightly flattened at the apex, 55–60 × 40–50 mm and a pseudostipe, 40–50 × 20–25 mm, narrowing toward the base, ≤10 mm; peridium yellowish olivaceous, very thin (<1 mm), showing pseudoperidioles as black ellipsoid patches.

Basidiospores globose, 8.4–11.6 µm, in average 9.9 µm (including spines); spines 1.2–2.3 µm (average = 1.6 µm) long, more or less isolated.

Specimen examined: **MACEDONIA, Skopska Crna Gora Mountain**, 800–900 m a.s.l., in meadow close to deciduous forest (*Quercus* spp., *Carpinus orientalis*, and planted *Castanea sativa*), 9-Oct-2010, leg. K. Rusevska (05MCF5506, LK024184).

Pisolithus arhizus (Scop.) Rauschert, Z. Pilzk. 25: 51. 1959. Fig. 3

Basidiomes with subglobose to ellipsoid head, 30–60 × (40–)55–90 mm tapering into irregular pseudostipe ≤95 mm × (20–)25–40(–50) mm, with narrow base ≤10 mm diam; peridium pale ochre to brown black.

Basidiospores globose, 8.4–11.6 µm, in average 9.9 µm (including spines); spines 1.2–2.3 µm (average = 1.2 µm) long, isolated but very compact, in parts forming small pyramids.

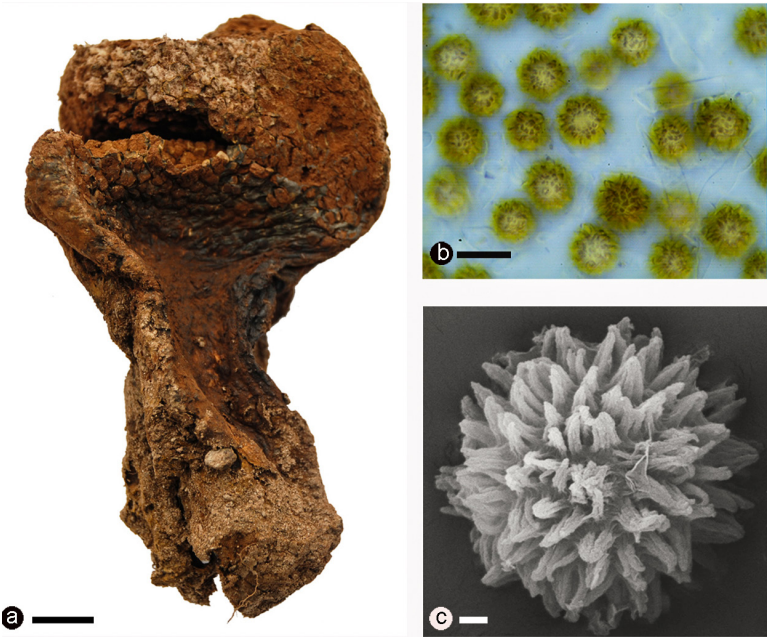

Fig. 2. *Pisolithus tinctorius* (05MCF5506). a. basidioma; b. spores (LM); c. spores (SEM). Scale bars: a = 1 cm; b = 10 μm; c = 1 μm.

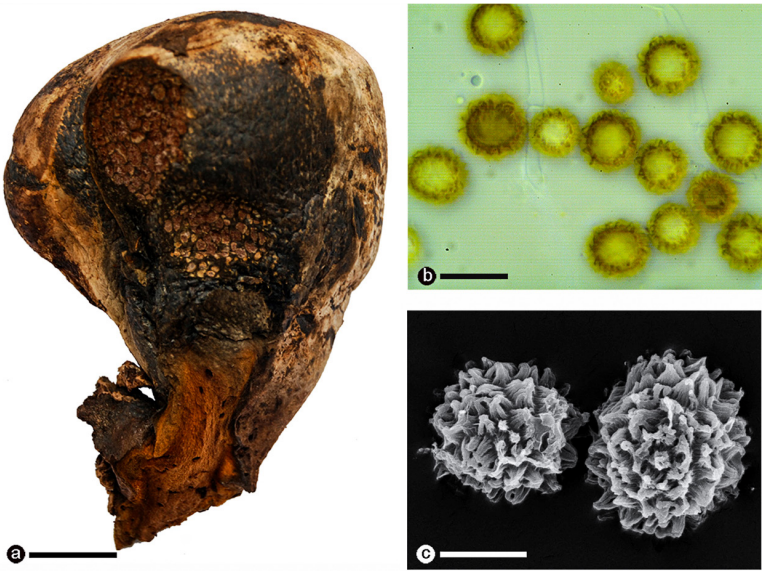

Fig. 3. *Pisolithus arhizus* (05MCF5324). a. basidioma; b. spores (LM); c. spores (SEM). Scale bars: a = 1 cm; b = 10 μm; c = 5 μm.

Specimens examined: **MACEDONIA**, **Galichica National Park**, between Oteshevo and Kale, 900–1000 m a.s.l., Macedonian oak forest, 8-Oct-1988, leg. M. Karadelev (02MCF9560, LK024180); Gradishte, at roadsides, 800 m a.s.l., grassy place in Macedonian oak forest, 10-Nov-2005, leg. K. Rusevska (05MCF5324, LK024181); **Pogana**, 300 m a.s.l., meadow in *Quercus coccifera* forest, 28-Oct-1987, leg. M. Karadelev (87MCF7741, LK024178); **Skopska Crna Gora**, above Chucher-Sandevo vill., 600 m a.s.l., degraded oak forest (*Querco-Carpinetum orientalis*), 6-Oct-1998, leg. M. Karadelev (98MCF4331, LK024179).

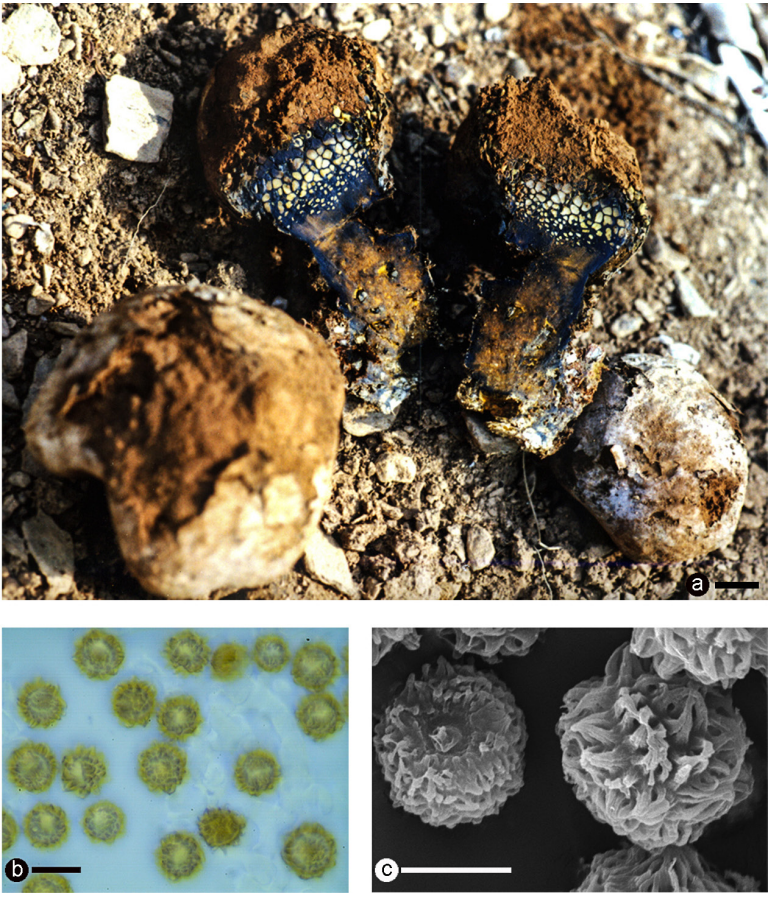

Fig. 4. *Pisolithus capsulifer* (03MCF3428). a. basidioma; b. spores (LM); c. spores (SEM). Scale bars: a = 1 cm; b = 10 µm; c = 5 µm.

Pisolithus capsulifer (Sowerby) Watling, Phosri & M.P. Martín, Mycotaxon 120: 202. 2012. Fig. 4

Basidiomes with globose to subglobose head, 25–35 × 45 mm, pseudostipe 40 mm long, 20–22 mm wide, and incorporating soil particles; peridium pale

ocher to black; PSEUDOPERIDIOLES dark yellow when cut, becoming darker and finally black on drying.

BASIDIOSPORES globose, 6.8–11.0 µm, in average 9.3 µm (including spines); spines 1.1–2.2 µm (average = 1.6 µm) long, mainly united in conical structures.

SPECIMENS EXAMINED: **MACEDONIA**, **BISTRA MOUNTAIN**, above Tresonche vill., 1100 m a.s.l., open place in deciduous forest, on cinnamon soil, 1100 m a.s.l., 3-Aug-2002, leg. K. Rusevska (02MCF3355, LK024182); 12-Jul-2003, leg. K. Rusevska (03MCF3428, LK024183).

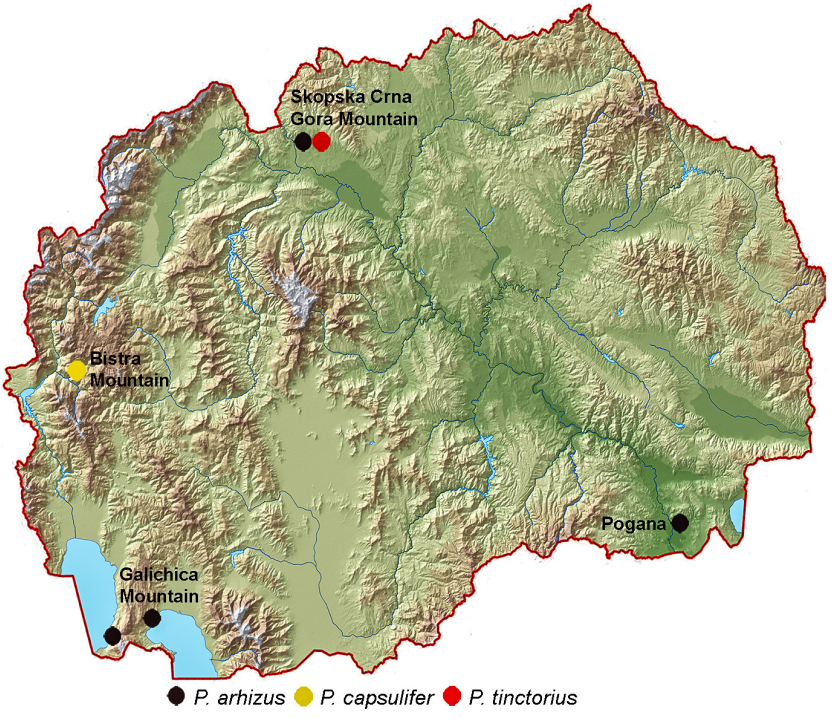

FIG. 5. Distribution map of *Pisolithus* collections from Macedonia analyzed in this study.

Discussion

The analyzed *Pisolithus* samples were obtained from four different localities in Macedonia: Pogana, and mountains Bistra, Galichica, and Skopska Crna Gora (FIG. 5).

Bistra Mountain is situated in western Macedonia. *Pisolithus capsulifer*, a new species for the mycobiota of Macedonia, was found near Tresonche village. The samples were collected at approximately 1000 m on cinnamon soil in an open area with common juniper shrubs. The site is close to a xerophytic and

thermophilic community of pubescent oak and hop hornbeam as dominant trees; *Acer monspessulanum* and *Fraxinus ornus* are also present. *Pisolithus capsulifer* is known from a few sites in three European countries (Sweden, France, and England) and Japan (Phosri et al. 2012).

Pogana, which lies in southern Macedonia, is one of three *P. arhizus* localities. The dominant forest species, evergreen shrub kermes oak, forms a well-developed forest community (*Coccifero-Carpinetum orientalis*) together with *Quercus pubescens*, as well as *Phillyrea media,* the dominant evergreen shrub. Several other plant species also present are *Pistacia terebinthus*, *Fraxinus ornus*, *Coronilla emeroides*, *Colutea arborescens*, *Crataegus heldreichii*, *Lonicera etrusca*, and *Cistus villosus.*

Galichica Mountain is situated between two lakes in southwestern Macedonia. *Pisolithus arhizus* is known from two mountain sites (one on the Lake Ohrid side and the other on the Lake Prespa side) between 800 and 1000 m altitude. The collections were made in Macedonian oak forest with *Quercus trojana* dominant and *Q. cerris, Q. pubescens, Carpinus orientalis,* and *Fraxinus ornus* also present.

The mountain Skopska Crna Gora lies in northern Macedonia. Two *Pisolithus* species were found on different sites in oak-hornbeam forests. *Pisolithus arhizus* was collected at 600 m from a degraded forest, while *P. tinctorius* was recorded at 800 m in a well-developed association mixed with planted sweet chestnut trees.

Using ITS barcodes (see Schoch et al. 2012) to analyze fungi has greatly clarified the *Pisolithus* complex in Macedonia. Our study confirms the presence of three *Pisolithus* species in the country: *P. arhizus, P. capsulifer,* and *P. tinctorius*, all growing near oak trees in deciduous forests. Thus far, *P. capsulifer* appears specific to a single locality and substrate (terra rossa on silicate ground). *Pisolithus arhizus,* as noted, is already included in the National Red List of Fungi. The two new species for Macedonia, *P. capsulifer* and *P. tinctorius*, are also rare and should be protected.

As *P. tinctorius* and *P. arhizus* have the same spore size and *P. arhizus* and *P. capsulifer* have a similar peridium colors, it is almost impossible to separate these species morphologically. In the face of such similar microscopic and macroscopic characters, we do not propose a key here.

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